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Antioxidant potentials of successive green solvent extracts from the unexplored Ficus subincisa

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Abstract:

Ficus subincisa Buch-Ham.Ex Sm. is considered an endemic medicinal plant. The current research based on the antioxidant potential of four different green solvent extracts obtained by the successive solvent extraction method from the unexplored *F.subincisa* leaves was evaluated. Various in vitro assays were performed to achieve the aim and variation in scavenging potential of the four green extracts, showed the highest total phenolic content (TPC), Total flavonoid content (TFC), and free radical scavenging potential against DPPH, ABTS, FRAP Phosphomolybdate, and nitric oxide radical scavenging assay as compared to other extracts.The extraction of *Ficus subincisa* with different green solvents namely: d-limonene, isopropyl alcohol, ethyl lactate, and hydroalcohol.Extracts were evaluated for their antioxidant activity using different assays namely, DPPH, ABTS, and nitric oxide scavenging. The Folin-Ciocalteau and $AlCl_3$ colorimetric methods were measured the content of total phenolics and total flavonoids respectively. Reducing power was determined by phosphomolybdate and Ferric reducing antioxidant power (FRAP) methods.Considerable amount of phenolic and flavonoid contents were recorded in the hydroalcohol extract. Although hydroalcohol fractions exhibited good antioxidant activities, the most distinguished radical scavenging potential. Hydroalcohol sowed equivalent to standard in radical scavenging activity by DPPH. Hydroalcohol showed the higher radical scavenging activity by DPPH (92.03%), Nitric oxide (89.21%) and by ABTS (89.45%), antioxidant activity, the highest total phenols content (78.67 ± 1.07 mg GAE/g fraction), the highest total flavonoids content (195.58 ± 3.31 mg quercetin equivalent/g extract fraction). The hydroalcohol extract showed a reducing power of (93.2%) and (1373.91 ± 4.35 µM/ml Ascorbic acid equivalent) using the phosphomolybdate and FRAP assays, respectively.Hydroalcohol shows the strongest antioxidant activity, and it can be attributed to its high content in phenolic and flavonoid compounds. It can be concluded that leaves of *Ficus subincisa* can be used as an effective natural source of antioxidant, and as a commercial basis for the evolution of nutraceuticals

Keywords: *Ficus subincisa*, green solvent extracts, Total phenol content, total flavonoid content, Free radicals, Antioxidant activity.

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1. Introduction

Free radicals having one or more unpaired electrons are highly reactive atoms or groups of atoms. Reactive oxygen species (ROS) are continuously generated inside living organisms by different mechanisms, this is the potency of reactive free radicals containing oxygen(1).In mitochondria, ROS are produced internally as a normal part of metabolism, whereas external factors such as environmental adulterator, smoking radiation, drugs, insecticide, hazardous solvents, and ozone also stimulate the production of ROS as well as Reactive nitrogen species (RNS)(2). Intracellular defense mechanisms are present to balance the production and neutralization of free radicals and ROS in all organisms. The disparity between antioxidant defenses and free radical production leads to a deluge of ROS and the cells bear the effect of oxidative stress. One of the prime procedures affected in several diseases such as carcinoma, diabetes mellitus, aging, and neurodegenerative disorders is the antioxidant mechanism that is developed for the protection of living systems against oxidative injuries caused by reactive oxygen and nitrogen species(3). By overcoming oxidative stress, antioxidants are needed, to maintain the normal physiological function of cells. At low concentrations of antioxidants, the substance is present, donates own electrons to ROS, and neutralizes the adverse effects of the ROS(4). However, essential antioxidant mechanisms can be ineffective, so dietary intake of antioxidants becomes important. In malice of the wide use of artificial antioxidants in food processing, the natural antioxidants from plant resources have acquired special intrigue. The antioxidant activity of plants is associated with the various phytochemicals present in the plant extracts such as phenols, flavonoids, carotenoids, anthocyanins, tannins, and terpenes and phenolic/flavonoid compounds attribute more to the biological activity of plant materials(5).

The plant *Ficus subincisa* is a member of the Moraceae family and is commonly known as the mulberry family or Fig family. It widely exists in Vietnam, India, and Southeast Asian countries. In India, the Ficus family has been used as a traditional medicine for the treatment of inflammation, skin diseases, headache, and sharp pain due to rheumatism(6). Recently, much attention has been paid to Ficus species due to their diverse biological activities and ethnopharmacological significance. Despite this, investigation related to the antioxidant activity is still not explored(7). Given this background, the present study aimed to evaluate the antioxidant activity of *F.subincisa* leaves extracts isolated from four different green solvent extracts. Therefore, this work aimed to test the potential use of different green solvents, and also the purpose of this study was to evaluate different extractives of leaves of *F.subincisa* as new potential sources of natural antioxidants and phenolic compounds. Antioxidant activities of different extracts of the plant were also evaluated by using DPPH free radical scavenging assay, Ferric reducing the ability of plasma, Phosphomolybdate assay, ABTS radical scavenging, and nitric oxide radical scavenging assay.

The worldwide chemical industry plays a vital role in important technological and scientific fields associated with the future of sustainable development in developed and developing countries. From the beginning, the leaders of the major chemical industries participated in the debate on the actions and changes needed to achieve the goals of Sustainable Development and identified their share of responsibility towards these goals(8).In chemical fields, researchers are paying attention to subsist to sustainability challenges to minimize potential environmental and health implications of various chemicals used by them during laboratory work(9). In Modern times green chemistry has advanced for a great variety of research providing pioneering research and practical applications for a wide spectrum of chemical products. Among other things, the reduction of global warming and the use of green solvents(10).

In this work, the potential use of different green solvents, namely hydroalcohol,d- limonene, isopropyl alcohol, and ethyl lactate to the extraction of *Ficus subincisa*. Ethyl lactate and limonene are environment-friendly solvents, easily biodegradable, with polarities in the range of acetonitrile and hexane, respectively. Both solvents are accepted as GRAS (generally recognized as safe) and approved by the U.S. Food and Drug Administration as medicament and nourishment supplement.

2. Materials and methods

2.1 Chemicals and reagents

Gallic acid, ascorbic acid, quercetin, potassium ferricyanide (III), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2-azinobis(3-ethylbenzothiazoline)-6 sulphonic acid), TPTZ(2,4,6-tripyridyl-s-triazine), purchased from Sigma-Aldrich. Folin–Ciocalteu reagent, potassium persulfate, sodium carbonate, EDTA(ethylenediaminetetraacetic acid), sodium

nitroprusside, sulphanilamide, and all solvents were procured from Merck Co. and Hi-Media Pvt. Ltd. d-limonene (Lobachemie), ethyl lactate (Lobachemie), ethanol (Merck), isopropyl alcohol (Merck. All other reagents were of analytical grade. UV-Visible spectrophotometer (Systronics 118) was used.

2.2 Collection of plant material and sample preparation

Leaves of *F.subincisa* were collected from the Tehri Garhwal regions of Uttrakhand. The plant was identified and authenticated with the help of the department of Botanical Survey of India and Voucher specimens (N0-31, 02/2019) of the plants were deposited in the herbarium center of Department of Botanical Survey of India, Dehradun, Uttrakhand, India. The leaves of *F.subincisa* were cleaned under tap water followed by distilled water and dried in a hot air oven at 40^0C. Dried samples were ground and stored at 4^0C until extraction. The extraction was carried out in a using flat bottom flask attached to water condenser is placed on the magnetic stirrer(11), successively using solvents in increasing order of polarity d-limonene, isopropyl alcohol, ethyl lactate, and hydroalcohol up to 4 h at 40^0C. The extracts were concentrated under reduced pressure in a rotary evaporator and stored at 4^0C. For *F.subincisa* the extraction yields were 22.19%, 6.147%, 9.93%, and 26.11% for d-limonene, isopropyl alcohol, ethyl lactate, and hydroalcohol, respectively. Without the interference related to the overall (and variable) water content, the extractions were carried out on dry material to obtain reproducible results. All data herein reported are referred to as plant dry weight, as generally done when plant extractions were carried out.

2.3 Determination of total bioactive components

2.3.1 Total phenolics content

The total phenolic content was determined by employing the methods given in the literature with slight modification. Sample solution (1 mL) was mixed with 10% Folin-Ciocalteu reagent (1.5mL) and shaken vigorously. Then all vials were kept in a dark place for 5 min.Then Na_2CO_3 solution (1.5 mL, 5%) was added.Again all the vials were kept in the dark for 2h. The sample absorbance was read at 760nm(12). The total phenolic content was expressed as equivalents of gallic acid according to the equation obtained from the standard gallic acid graph.

2.4 Total flavonoids content

The total flavonoid content was determined using the modified method given in the literature. Briefly,extract solution (1mL) was mixed with distilled water(3ml),sodium nitrate (0.3 ml,5%) and the same volume of aluminium trichloride (0.3 ml,10%) in methanol. Similarly, a blank was prepared. The samples and blank absorbances were read at 415 nm after incubation at room temperature (25°C ± 1°C) for 10 minutes. The total flavonoid content was expressed as equivalents of Quercetin according to the equation obtained from the standard quercetin graph(13).

2.5 Radical scavenging activity

2.5.1 Free radical scavenging activity (DPPH)

The effect of the extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was carried out to the standard method with minor modification. A stock solution of DPPH was prepared by dissolving 0.012 gram of DPPH in 50 ml of methanol. The working solution was prepared by diluting the stock solution to get an absorbance between 0.9 to 1.2.3 ml of the working solution was added to 1 ml of the diluted plant sample (1 mg/ml). The reaction mixture was mixed properly and incubated in the dark for 30 min. Then the absorbance was taken at 512 nm using ascorbic acid as standard(14).

2.6 ABTS (2,2'- azino-bis-3-ethylbenzothiazoline-6-sulphuric acid) radical

2.6.1 cation scavenging activity

ABTS radical scavenging activity was assessed by the standard method with slight modification. Reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to stand for 12-16 in dark at room temperature, $ABTS^+$ radical cation was produced. Before beginning the assay, the ABTS solution was diluted with methanol to an absorbance of 0.70±0.02 at 734 nm. An aliquot solution (0.2 mL) was added to the ABTS solution (2 mL) was mixed properly and incubated in dark for 30 min. Then sample absorbance was read at 734 nm using gallic acid as standard(15).

2.7 Nitric oxide (NO) scavenging assay

Nitric oxide (NO) scavenging activity was carried out with slight modification. At physiological pH Sodium nitroprusside in an aqueous solution spontaneously generate nitric oxide, which interacts with oxygen to produce nitrite ions determined by the use of Griess reagents. A volume of 10 mM of 2ml sodium nitroprusside and phosphate buffer of 0.5 ml saline (pH 7.4) was mixed with plant extract of 0.5 ml and ascorbic acid individually at various concentrations (1000-100 µg/ml). The reaction mixture was incubated for 180 minutes at 25 °C. After that incubated solution of 0.5 ml was withdrawn and mixed with 1 ml of sulfanilic acid reagent (0.33 in 20% glacial acetic acid) and allowed to stand for 5 min at room temperature for completing diazotization. Then 0.1% w/v of 1ml naphthyl ethylenediamine dihydrochloride was added, then the

mixture was incubated at room temperature for 30 min. The absorbance was taken at 540 nm(16).

2.8 Reducing power

2.8.1 Ferric reducing antioxidant power (FRAP) method

The FRAP assay was carried out as described in the literature with slight modification. Sample solution (0.1 mL) was added to premixed FRAP reagent (2 mL) (containing ferric chloride 20 mM , (TPTZ) 10 mM in 40 mM HCl, acetate buffer 0.3 M , pH 3.6 in a proportion of (1: 1: 10). Then, the sample absorbance was read at 593 nm after incubation at room temperature (25°C ± 1°C) for 30 minutes. Absorbance was taken at 593 nm taking ascorbic acid as standard(17).

2.9 Phosphomolybdenum method

The phosphomolybdenum method was evaluated according to given in literature with slight modification. An aliquot (0.3 mL) was combined with 3 mL of reagent solution (28 mM sodium phosphate, 4 mM ammonium molybdate, and 0.6 M sulphuric acid). and incubated in a water bath at 70°C for 90 min. Then the absorbance was measured against a blank at 695 nm after samples cooled down to ambient temperature(18).

3. Result and Discussion

3.1 Total Phenolics and Flavonoids

Polyphenols are the most abundant antioxidants in the plant kingdom and the antioxidant capacity of extract might likely be due to these phenolic compounds. Figure 1. summarizes the total phenolic contents expressed as gallic acid equivalents (GAEs), ranging from 11.4 to 78.67 in *F.sunincisa*.Observed phenolic content of different extracts follows the order Hydroalcohol > Ethyl lactate > Isopropyl alcohol > d-limonene. The maximum quantity of total phenol content was observed in Hydroalcohol, 78.67 ± 1.07 mg GAE/g fraction, with a significant difference with the rest of the fractions, followed by Ethyl lactate and Isopropyl alcohol with 46 ± 0.86 mg GAE/g fraction and 35 ± 0.91 mg GAE/g fraction, respectively. d-limonene showed the lowest content of phenols with11.34 ± 0.37 mg GAE/g.

Flavonoids are the most common group of polyphenolics compounds in the human diet. They are abundant in plants. Flavonoids show several antioxidants, antiviral, and antimutagenic effects. For example, quercetin is a well-known plant-derived flavonoid that may have anti-inflammatory and antioxidant properties(19). The total flavonoid content varied from 195.58± 3.31 to 24.42 ± 2.24 mg quercetin equivalent/g extract/fraction The total flavonoid content of *F.subincisa* extracts is presented in figure 2. Reported flavonoids content of the different extracts follows the order Hydroalcohol > Ethyl lactate > Isopropyl alcohol > d-limonene. The maximum quantity of total Flavonoid content was observed in Hydroalcohol, 195.58 ± 3.31 mg quercetin equivalent/g extract, with a significant difference with the rest of the fractions, followed by Ethyl lactate and Isopropyl alcohol with 133.23± 2.21 mg quercetin equivalent/g extract fraction and 43.82 ± 1.81 mg quercetin equivalent/g extract fraction, respectively. d-limonene showed the lowest content of flavonoid with 24.42 ± 2.24 mg quercetin equivalent/g extract. The maximum flavonoids and phenolic content were obtained for the hydroalcohol extracts (195.58 mg quercetin equivalent/g extract)

Fig.1-Total phenolic content

and (78.67 GAEs/g extract) in *F.subincisa*.

3.2 Determination of antioxidant activity

Antioxidant properties of different plant aliquots can be evaluated using various in vitro assays. Antioxidant assays in a biological system can assess reducing power or can measure free radical scavenging ability. For measuring free radical scavenging ability, methods are grouped into two groups, the chemical reaction involved according to single electron transfer and hydrogen radical transfer(20). Single-electron transfer based-methods detect the ability of an antioxidant to reduce any compound to transfer one electron, including metals and radicals. Methods based on this concept include

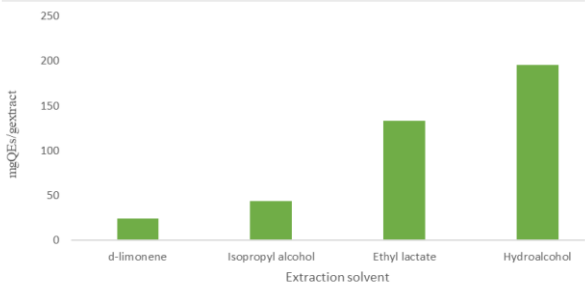

Fig.2-Total flavanoid content

a substrate oxidant that abstracts an electron from the antioxidant, causing a change in the color of the

substrate. The degree of color change is proportional to the antioxidant concentrations(21).

3.2 Free Radical Scavenging Assays

3.2.1 DPPH radical scavenging capacity

2,2-diphenyl-1-picrylhydrazyl (DPPH), in solution, is a stable free radical, which has been widely accepted as a tool for estimating free radical scavenging activities of antioxidants. This method is simple and highly sensitive. The DPPH assay is mainly based on an electron transfer reaction. The amount of $DPPH^{\cdot}$ in the antioxidant test system has been reported in various methods, however, monitoring with UV spectrometer has become the most widely used. The reduction capability of DPPH radical is measured by the decrease in absorbance at 517 nm induced by antioxidants Then, color changing from purple to yellow is the consequence of the reducing ability of antioxidants toward DPPH. The higher the discoloration of the DPPH hydroalcohol solution, the lower the absorbance of the reaction mixture, indicating thereby significant free radical scavenging capacity(22). The results have been reported as IC50, which is the amount of antioxidant necessary to decrease the initial $DPPH^{\cdot}$ by 50% expressed in µg/mL. The results of DPPH scavenging activity of hydroalcohol extract and *F.subincisa*, are summarized in figure 3. Overall, the IC50 of DPPH free radical scavenging capacity of four samples were found between 70.46 ± 0.28 to 740.34 ± 0.44 µg/mL. d-limonene, isopropyl alcohol, and ethyl lactate showed comparatively strong DPPH free radical scavenging capacity. Hydroalcohol showed equivalent to Ascorbic acid which is a standard.

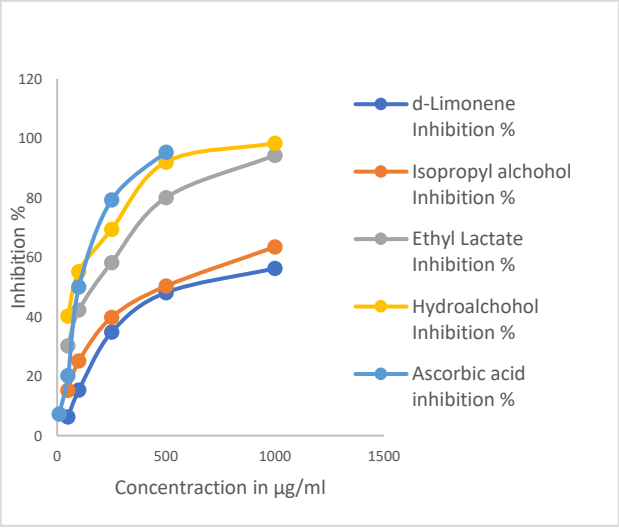

Fig.3-Radical scavenging activity of F. subincisa extracts measured by DPPH method

Similarly, ethyl lactate and isopropyl alcohol showed moderate activity. Figure 3 shows the dose-response curves of DPPH radical activities of extracts from *F.subincisa*. It was reported that all samples exhibited potent scavenging activities in a concentration-dependent manner. At a concentration of 500 µg/mL, the scavenging activities of Hydroalcohol reached a plateau of 92.03% while, at the same concentration, the scavenging effects of ethyl lactate, isopropyl alcohol, and d-limonene were 80.07%, 50.28%, and 48.12% respectively.

3.2.2 ABTS assay

ABTS radical scavenging activity involves a more dire, radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant aliquots, beverages, and biological fluids. The actual version of this assay, a stable $ABTS^{+}$ radical cation that has a blue-green chromophore absorption, was produced by oxidation of ABTS with potassium persulfate. The antioxidant activity of the plant extracts is determined by the discoloration of the ABTS, by measuring the reduction of the absorbance at 734 nm. Gallic acid as the reference standard, the adjunct of discoloration, expressed as percentage inhibition of $ABTS^{+}$ is determined as a function of the concentration(23).

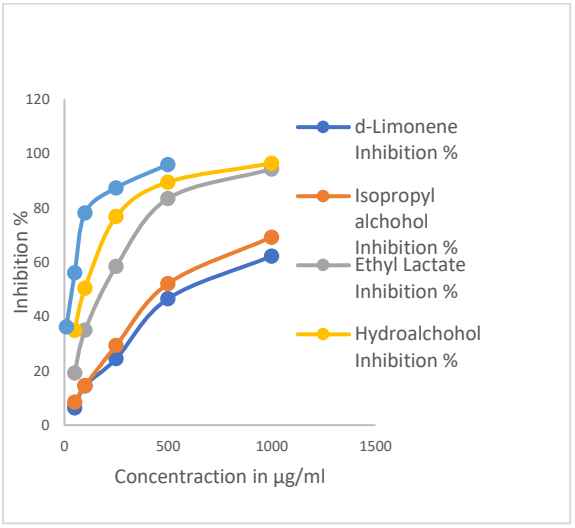

Fig.4-Radical scavenging activity of F. subincisa extracts measured by ABTS method

The results of the ABTS scavenging activity of hydroalcohol extract and its partitioned fraction of *F.subincisa*, are summarized in figure 4. Overall, the IC50 of ABTS free radical scavenging capacity of four extracts was found between 98.76 ± 1.09 to 712.33 ± 1.70 µg/mL. hydroalcohol and ethyl lactate showed comparatively strong ABTS free radical scavenging capacity. Similarly, isopropyl alcohol showed moderate activity. Figure 4 shows the dose-response curves of ABTS radical activities of extracts from *F.subincisa*. It was found that the radical scavenging activities of all extracts increased with the

concentration. All samples exhibited potent scavenging activities in a concentration-dependent manner. At a concentration of 500 µg/mL, scavenging activities of hydroalcohol reached a plateau of 89.45% while, at the same concentration, scavenging effects of ethyl lactate, isopropyl alcohol, and d-limonene were 83.39%,52.12%, and 46.58% respectively. While at the same concentration the activity of gallic acid was 95.89%. The antioxidant activity of *F.subincisa* and its fractions and standard Gallic acid increased in the following order as Gallic acid > Hydroalcohol > Ethyl lactate > Isopropyl alcohol > d-limonene.

3.2.3 Nitric oxide (NO) scavenging activity

Nitric oxide (NO) is a reactive free radical produced from amino acid L-arginine by vascular endothelial cells, phagocytes, and certain brain cells. Because unpaired electron nitric oxide is classified as a free radical, displays important reactivity with a certain type of protein other than free radical(24). The level of NO· was significantly reduced in this study by the hydroalcohol extracts of leaves of *F. subincisa* (with an IC50 value of 424.63 µg/ml) and its ethyl lactate extract partitioned (with an IC50 value of 443.34 µg/ml) both of which are comparable to that of ascorbic acid (with an IC50 value of 99.98 µg/ml) shown in Fig.5. NO scavenging capacity of the extract may help to arrest the chain of reactions

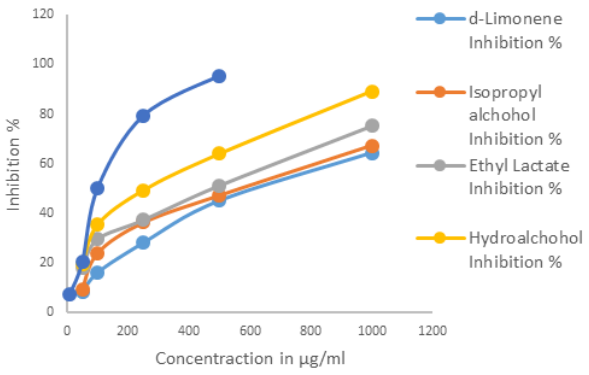

Fig.5-Radical scavenging activity of F. subincisa extracts measured by Nitric oxide method

initiated by excess generation of NO that lead to various pathogenic pathways underlying a large group of disorders including muscle diseases, inflammatory bowel disease, sepsis and septic shock, primary headaches, and stroke. Additionally, evidence shows that NO regulates neurotoxin-induced cell damage and is involved in neuronal cell death in Parkinson's disease and other neurodegenerative disorders such as Alzheimer's disease. Potential NO scavenging activity of the plant may explicate the use of it for the treatment of inflammatory and neurological disorders. The observed sequence can be ranked nitric oxide scavenging ability as Hydroalcohol > Ethyl lactate > Isopropyl alcohol > d-limonene. The d-limonene extracts from the *F.subincisa* exhibited the lowest radical scavenging activities when reacted with these radicals scavenging methods.

3.2.4 FRAP assay

Ferric reducing antioxidant power assay is based on reductants in a redox-linked colorimetric method employing as easily reduced oxidant, Fe(III) in the presence of TPTZ (2,4,6-tris-(2-pyridyl)-s-triazine), forming an intense blue Fe-TPTZ complex can be monitored by measuring absorbance maximum at 593 nm. Decreased absorbance is proportional to the antioxidant content(25). The FRAP assay results of the green solvent extracts expressed ferric reducing antioxidant power (µM/ml)in figure 6. Hydroalcohol showed the highest reducing power with 1373.91 ± 4.35 µM/ml Ascorbic acid equivalent, followed by ethyl lactate with 1002.25 ± 7.90 µM/ml Ascorbic acid equivalent. FRAP values varied from 1373.91 ± 4.35 to 207.25 ± 6.34 µM/ml Ascorbic acid equivalent. The ferric-reducing antioxidant power of *F.subincisa* extract and Ascorbic acid increased in the following order Ascorbic acid > Hydroalcohol > Ethyl lactate > Isopropyl alcohol > d-limonene.

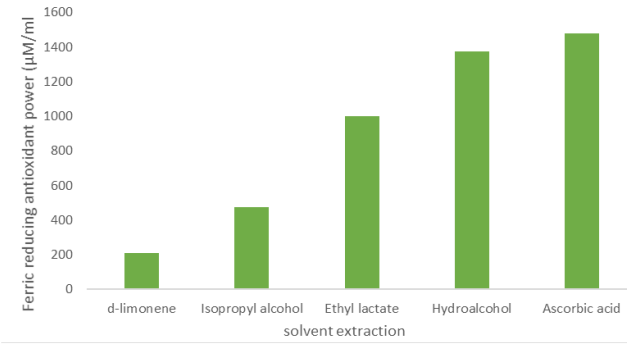

Fig.6-Ferric reducing antioxidant potential assay

3.2.5 Phosphomolybdate assay

The total antioxidant potential of *Ficus subincisa* extracts was estimated. It was observed that the reduction of Mo (VI) to Mo (V) complex which is green in color with maximum absorption at 695 nm. Increased absorbance of the reaction mixture is directly proportional to increased total antioxidant capacity(26). In the present assay, all the different fractions showed a good total antioxidant index, which was concentration-dependent. The results of reducing the power of *F. subincisa* extract and its

partitioned fraction are summarized in figure 7. Overall, the IC50 of the four samples were found between 744.06 ± 2.09 to 130.16 ± 3.13 µg/mL. Figure 7 shows the dose-response curve of the total antioxidant capacity of extracts from Ficus subincisa. It was found that all test samples exhibited potent activities in a concentration-dependent manner. At a concentration of 500 µg/mL, the antioxidant activity of *F.subincisa*, its fractions, and gallic acid increased in the following order Gallic acid > Hydroalcohol > Ethyl lactate > Isopropyl alcohol > d-limonene.

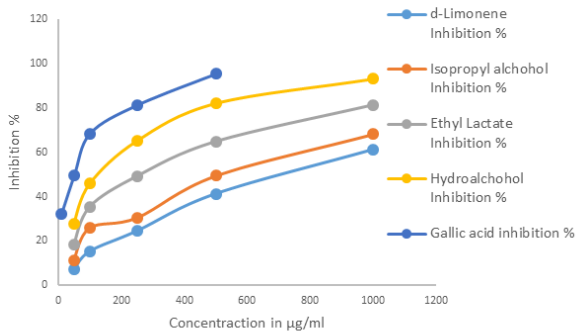

Fig.7-Reducing power of F. subincisa extracts measured by Phosphomolybdate method

4. Conclusion

In conclusion, we hope the representative of green solvent extraction illustrated in this research paper has provided incentives for further work in developing green pharmaceutical processes. Eventually, there will be no need to emphasize green chemistry to pharmaceutical chemists as it will become the natural course of action when the ultimate good to the company, patient, and environment is considered. This is the first study on the antioxidant activities of different green solvent extracts of uninvestigated *Ficus subincisa*. The biocompatible extracts were analyzed to obtain the total phenolics, and flavonoids contents, and additionally, these extracts were tested to evaluate their antioxidant abilities. Among the four extracts, hydroalcohol extracts showed higher inhibition activity in both tested extract samples. In current times, the prevention of malignant tumor or cancer diseases could also be related to the ingestion of fresh vegetables, fruits, or teas rich in antioxidants from natural products, and the herein reported results could be valuable to consider these species as a candidate for preparing new food supplements and may represent a good starting model to the development of new drug formulations.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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